

THE CONVERSION OF L-HISTIDINE TO GLUTAMIC ACID BY LIVER ENZYMES*

BY ADOLPH ABRAMS† AND HENRY BORSOOK

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California)

(Received for publication, March 17, 1952)

Edlbacher and Neber (1) showed in 1934 that the liver enzyme named histidase degrades histidine to NH_3 , formic acid, and an unknown product which on further treatment with strong alkali yields glutamic acid. This led to the suggestion that glutamic acid is a metabolic product of histidine, a suggestion that was supported by the finding that glycogen was formed from histidine about as well as from glutamic acid (2). These findings did not prove that glutamic acid was one of the products of histidine metabolism, and the idea became questionable when the evidence from subsequent investigations with non-isotopic histidine (3), imidazole- N^{15} -histidine (4), and carboxyl- C^{14} -histidine (5) were negative or inconclusive.

In studies on the fate of carboxyl- C^{14} -L-histidine in the liver of rabbits after injection and after incubation with guinea pig liver slices, we have found direct evidence that glutamic acid is a major product of histidine metabolism. Another highly radioactive compound was isolated by ion exchange chromatography, whose properties with respect to chromatography and lability to alkali and acid appear to correspond to those reported for isoglutamine. Takeuchi (6) isolated and identified isoglutamine as a product of the action of urocanicase on urocanic acid, which was obtained by the action of another liver enzyme on histidine. The formation of isoglutamine as an intermediate is consistent with our finding that the label in the radioactive glutamic acid formed from carboxyl- C^{14} -histidine is not in the α -carboxyl group, and the inference is very strong that the label is in the γ -carboxyl group.

Materials and Methods

The synthesis of the carboxyl- C^{14} -L-histidine used has been described (7). Its specific activity was 23,000 c.p.m. per mg.

The non-protein filtrates of liver were obtained as follows. The liver

* These studies were aided by a contract between the Office of Naval Research, United States Navy Department, and the California Institute of Technology, Division of Biology (NR-164-304). The C^{14} used in this investigation was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

† Eli Lilly Research Fellow.

was homogenized in ice water and the protein precipitated and then washed with 5 per cent trichloroacetic acid, after which the combined original filtrate and washings were extracted with ether, evaporated to dryness *in vacuo*, and the residue taken up in 2 ml. of 1.5 N HCl. A small amount of insoluble material was discarded. The solution was then chromatographed on Dowex 50 with three strengths of HCl as eluent according to the method of Stein and Moore (8). The chromatographic column was water-jacketed and kept at 3–4° to minimize changes due to the relatively strong acid during the chromatography. The eluate was collected in 2 to 5 ml. fractions; 0.4 ml. aliquots of each fraction were neutralized and analyzed for ninhydrin-reactive material by the method of Moore and Stein (9); the radioactivity of each fraction was determined by means of a Geiger-Müller end-window counter on 0.6 ml. aliquots dried on Tygon painted aluminum cups.

Other details of procedure are given below with the experimental results.

Experimental Procedure and Results

In Vivo Experiment—20 mg. of radioactive histidine were injected into the ear vein of each of two rabbits and the livers were removed 40 minutes later in one case and 20 minutes later in the other. Figs. 1 and 2 give the distribution of radioactivity and ninhydrin-reactive material in the fractions of the eluate after chromatography of the non-protein filtrates.

In Vitro Experiment—3 mg. of radioactive histidine were incubated in three beakers, each containing eight guinea pig liver slices (a total of 677 mg. of dry weight of protein) and 4.0 ml. of Krebs-Henseleit Ringer's solution (10) under 95 per cent O₂ and 5 per cent CO₂ for 3.5 hours at 38°. At the end of the incubation the suspension of slices and saline was adjusted to pH 5 and immersed in a boiling water bath for 15 minutes and filtered. The filtrate was evaporated to dryness *in vacuo* and chromatographed as described above. The distribution of radioactivity is shown in Fig. 3.

Figs. 1, 2, and 3 show that a number of radioactive substances were resolved on the ion exchange resin. The areas of the radioactive peaks represent the relative amounts of radiohistidine and of each of its radioactive conversion products, formed in the liver. The identification of some of these as glutamic acid and aspartic acid in the *in vivo* experiment (Figs. 1 and 2) and of glutamic acid and of what appears to be an intermediate compound in the *in vitro* experiment (Fig. 3) is described in the following sections. The percentage conversion (Table I) of the histidine to glutamic acid and to the intermediate compound shows that this is a major pathway of histidine metabolism.

Isolation of Radioactive Glutamic Acid—The glutamic acid eluate frac-

tions shown in Figs. 1, 2, and 3 were identified by paper chromatography and paper electrophoresis. Coincidence of ninhydrin color and radioactivity indicated that the radioactivity was in the glutamic acid. Further evidence that the radioactive material in these fractions was glutamic

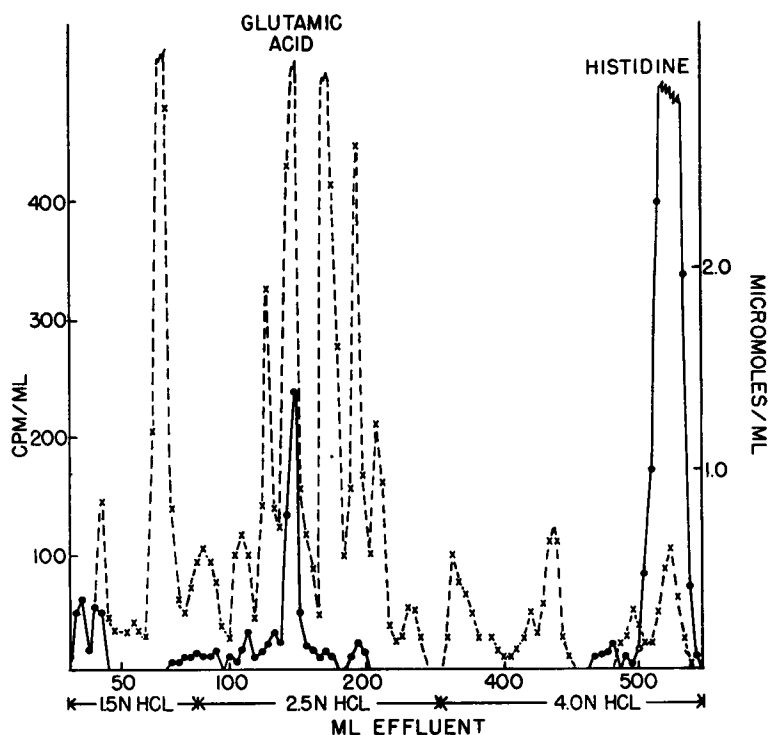


FIG. 1. Chromatographic analysis on Dowex 50 ion exchange resin of the non-protein fraction of rabbit liver 40 minutes after the injection of radiohistidine. The radioactivity of the eluate fractions, expressed as counts per minute per ml., is shown by solid lines. The ninhydrin reactivity of the same eluate fractions, expressed as micromoles per ml., is shown by dash lines. The peak of the histidine radioactivity, not shown, was 1400 c.p.m. per ml. and 1550 c.p.m. per ml. The radioactive glutamic acid and radioactive histidine are indicated on the diagram. The other ninhydrin peaks represent free amino acids and other ninhydrin-reactive material in liver; the other radioactive peaks are unidentified metabolic products of the radiohistidine.

acid was obtained by filter paper chromatography. There was only one ninhydrin spot. Its R_f values with propanol-ammonia and with phenol-water were identical with those of glutamic acid run simultaneously, and the radioactivity coincided exactly with the ninhydrin spot. This was conclusively established by carrier isolations and recrystallization to constant specific activity as follows. The fractions shown in Fig. 1 (*in vivo* experiment) and Fig. 3 (*in vitro* experiment) containing the supposed glu-

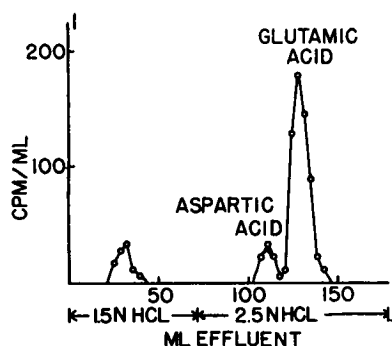


FIG. 2. Chromatographic analysis on Dowex 50 ion exchange resin of the non-protein fraction of rabbit liver 20 minutes after injection of radiohistidine. The radioactivity of the eluate fractions is expressed as counts per minute per ml. The radioactive glutamic acid and aspartic acid, derived metabolically from the radiohistidine (not shown), are indicated. The remaining radioactive peaks are unidentified metabolic products of the radiohistidine.

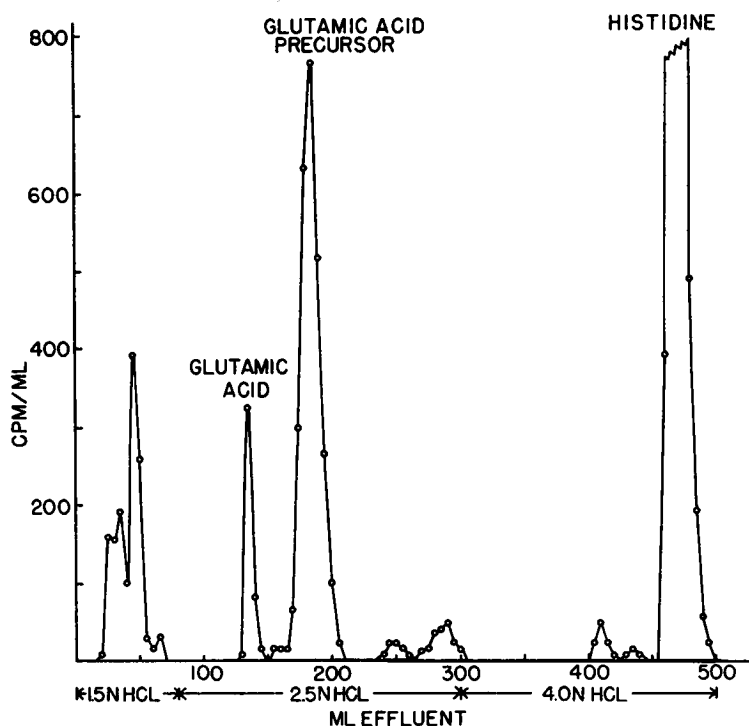


FIG. 3. Chromatographic analysis on Dowex 50 ion exchange resin of the non-protein fraction after 3.5 hours incubation of radiohistidine with guinea pig liver slices, in the presence of bicarbonate- CO_2 . The radioactivity of the eluate fractions is expressed as counts per minute per ml. Radioactive glutamic acid, a glutamic acid precursor, and radiohistidine from which they were metabolically derived, are indicated on the diagram. Note that aspartic acid, which is eluted just before glutamic acid (see Fig. 2), was not measurably radioactive. The remaining radioactive peaks are unidentified metabolic products of radiohistidine.

tamic acid were evaporated *in vacuo* to dryness, 100 mg. of non-isotopic glutamic acid were added in the first case and 80 mg. in the second, and they were recrystallized as the hydrochloride by saturation with HCl gas. Two recrystallizations gave specific activities of 4.4 and 4.3 c.p.m. per mg. in the one case and 8.8 and 8.3 c.p.m. per mg. in the second case.

Position of Label in Glutamic Acid—A 40 mg. (4.3 c.p.m. per mg.) and a 30 mg. (8.3 c.p.m. per mg.) sample of the carrier isolated 2 times recrystallized glutamic acid from the *in vivo* and the *in vitro* experiments, respectively, was treated with ninhydrin according to the method of Van Slyke *et al.* (11), and the liberated CO₂, which is derived from the α -carboxyl only, was collected as BaCO₃ whose radioactivity was then measured. In the first case (the *in vivo* experiment) only 10 per cent of the radioac-

TABLE I

Distribution of Radioactivity Derived from Carboxyl-C¹⁴-L-Histidine among Various Compounds of Liver Non-Protein Fraction

Tissue	Duration of experiment	Administered dose of carboxyl-C ¹⁴ -L-Histidine	Per cent total counts administered, found in compounds below			
			Histidine	Glutamic acid	Intermediate compound	Aspartic acid
Rabbit liver <i>in vivo</i>	40 min.	460,000 c.p.m.	6.4	0.58	Not identified	0.09
" " " "	20	460,000	Not determined	0.56	" "	0.09
Guinea pig liver slices	3.5 hrs.	67,140	51	3.0	20	None
" " " "	3.5	67,140	54	1.4	17	"

tivity was liberated by ninhydrin, and in the second case (the *in vitro* experiment) no radioactivity could be detected in the BaCO₃, although 5 per cent could have been measured. Quantitative recovery of the CO₂ was obtained.

The appearance of 10 per cent of the label of the glutamic acid in the α -carboxyl group in the case of the *in vivo* experiment was probably due to CO₂ fixation, the radioactive CO₂ having arisen by decarboxylation of the carboxyl-labeled histidine. Aspartic acid was labeled to a slight extent (see Figs. 1 and 2). This may have occurred either through C¹⁴O₂ fixation or conversion of γ -carboxyl-labeled glutamic acid. Proof that aspartic acid was labeled was established by rechromatographing the aspartic acid fraction from Dowex 50, which had been eluted with 1.5 N and 2.5 N HCl, on starch with 0.1 N HCl-propanol-H₂O. The radioactivity coincided exactly with the aspartic acid fraction. Labeled CO₂ incorporation has been shown to lead to labeling of the α -carboxyl of

glutamic acid exclusively (12-14); our results in the *in vivo* experiment indicate that 90 per cent of the label was very probably in the γ -carboxyl group.

This is shown more clearly in the *in vitro* experiment. The incubation was carried out in the presence of 0.024 M bicarbonate and under a continuous flow of 5 per cent CO_2 . This great excess of unlabeled CO_2 diluted the C^{14}O_2 arising from the histidine so greatly that no radioactivity was found either in the aspartic acid or in the α -carboxyl group of glutamic acid. Nevertheless, the isolated glutamic acid was highly radioactive. As the histidine was labeled in the 1 position, the inference is strong that the C^{14} was at the γ -COOH. Nevertheless, it must be stated that the last point has not been proved directly. It follows, then, that the amino group of histidine came off in the conversion (see below).

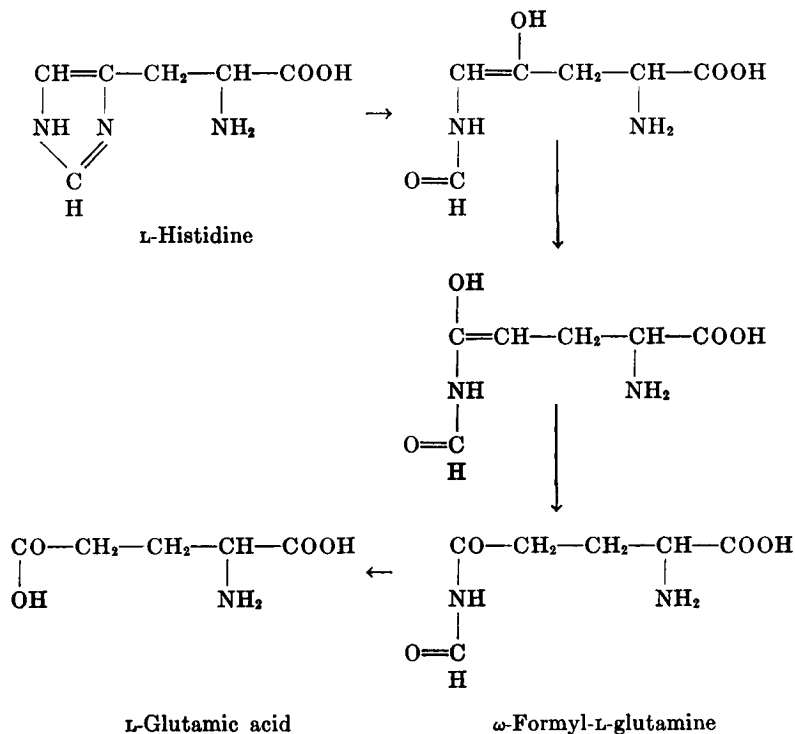
Isolation of Radioactive Precursor of Glutamic Acid—Fig. 3 shows a well resolved fraction which emerges after glutamic acid, and whose radioactivity is exceeded only by that of histidine itself. The fraction corresponding to the peak of this fraction was taken to dryness *in vacuo* at room temperature, and an aliquot was chromatographed on paper in a saturated phenol- H_2O system. The compound, detected by its radioactivity, had an R_F of 0.55. Only one radioactive spot could be detected. This spot did not produce a color with ninhydrin, possibly because the amount was not sufficient. After treatment with NH_4OH -water (1:1) at 100° for 2 hours, the compound was completely converted to one with an R_F of 0.3 corresponding to the position of glutamic acid. On the other hand, standing in 6 N HCl for 2 days caused less than 5 per cent conversion to glutamic acid, heating in 0.6 N HCl for 1 hour at 100° caused no significant change, and heating in 6 N HCl for 2 hours at 100° caused about 40 per cent conversion to glutamic acid. This behavior in acid and alkali corresponds to that reported for isoglutamine (6), and its R_F is close to that reported for glutamine (15). It is noteworthy that this compound was observed only in the extract from the *in vitro* experiment (Fig. 3); there was very little, if any, in the extract of liver from the whole animal (Figs. 1 and 2).

DISCUSSION

The argument that histidine was converted to glutamic acid enzymatically (and physiologically) rests on the fact that radioactive glutamic acid was isolated by relatively mild procedures from non-protein filtrates after presenting the systems studied with radiohistidine, while little or no radioactivity was found in the other common amino acids, including aspartic acid. The radioactive glutamic acid could not have arisen as an artifact in our manipulation of the liver extracts. The radioactive compound

which yields glutamic acid readily with alkali does so only very slowly with even strong acid. After the incubation the liver extracts were at no time above pH 5, and when they were treated with trichloroacetic acid it was in the cold and for a short time.

Edlbacher (1) proposed the accompanying mechanism for the conversion of histidine to glutamic acid.

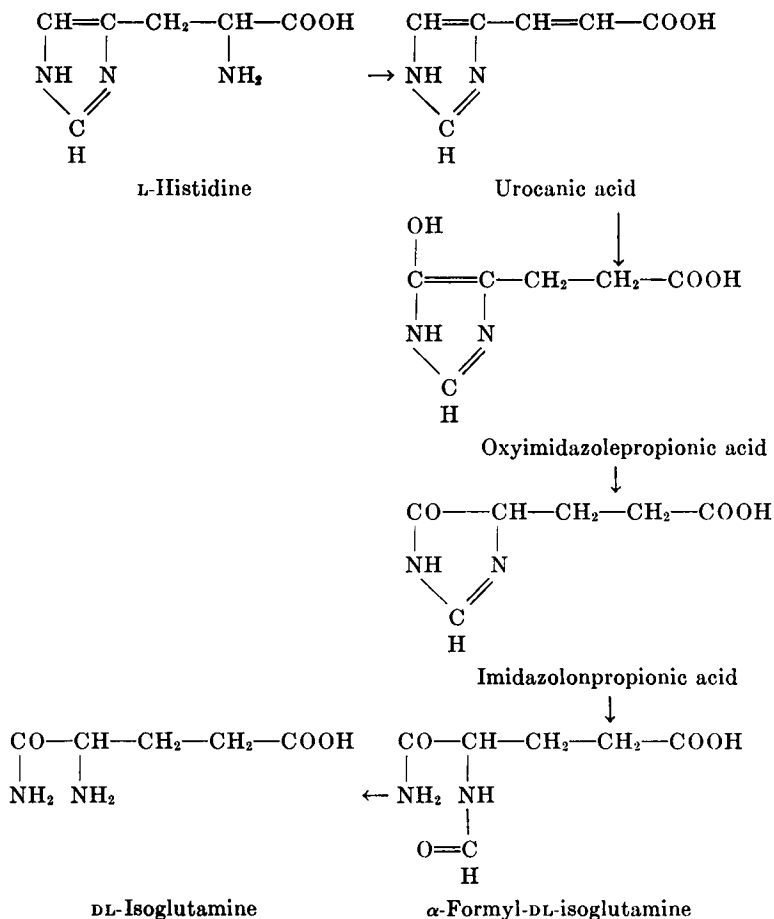


Oyamada (16) proposed a fundamentally different pathway for the enzymatic breakdown of histidine by liver enzymes. He isolated urocanic acid, α -formyl-DL-isoglutamine, and DL-isoglutamine, and proposed that this was the sequence in which these substances arose from each other. He did not designate glutamic acid as a physiological end-product. Oyamada's scheme is given in the diagram on the following page. Edlbacher did propose glutamic acid as the physiological end-product, its immediate precursor being glutamine. Glutamic acid can arise from either glutamine or isoglutamine, and evidence is presented here that glutamic acid is formed enzymatically.

Beginning with carboxyl- C^{14} -L-histidine, according to Edlbacher's mechanism the C^{14} would be entirely in the α -COOH of the glutamic acid

formed, while according to Oyamada's mechanism the C¹⁴ would be entirely in the γ -COOH group. Our evidence supports the latter mechanism and definitely eliminates that proposed by Edlbacher.¹

We have no evidence on the intermediates between histidine and isoglutamine proposed by Oyamada. Nor have we determined the optical



rotation of the glutamic acid we have found to be formed (enzymatically). Edlbacher isolated, after treatment of a precursor with alkali, L(+)-glutamic acid. If, as Takeuchi suggests, this precursor was DL-isoglutamine, the resulting glutamic acid should have been the DL form. The en-

¹ The formation of α -formamidinoglutaric acid through rearrangement of an intermediate product proposed by Walker and Schmidt (17), on the basis of indirect evidence, could also result in γ -carboxyl-labeled glutamic acid arising from carboxyl-labeled histidine.

zymatic hydrolysis of L-isoglutamine would, presumably, give only L-glutamic acid. This point needs to be investigated.

It remains to account for the inconclusive results obtained by D'Iorio and Bouthillier (5). These workers injected carboxyl- C^{14} -histidine into the rat and 4 and 18 hours later determined the C^{14} in the aspartic and glutamic acids, arginine, proline, and hydroxyproline in the carcass proteins. The label was present in all of these amino acids, most in the arginine, least in the aspartic acid. The authors concluded "that glutamic acid does not appear to be an important intermediate in histidine catabolism." The design of their experiments was such that direct glutamic acid formation from histidine could not have been shown. The time elapsed after the injection of the histidine was too long, 4 and 18 hours; the carcass protein rather than the non-protein fraction of the liver was analyzed. All that could be seen in their experiment was the general transfer of $C^{14}O_2$ arising from the decarboxylation of the histidine, as the highest degree of labeling in the protein arginine shows.

Tesar and Rittenberg (4) used imidazole- N^{15} -histidine and did not observe significant labeling of glutamic acid isolated from the proteins. They recognized that this negative result could have occurred as a result of the rapid deamination and reamination of glutamic acid and they pointed out that C^{14} labeling would be more conclusive.

After this work was concluded and the present communication written, a report appeared by Tabor and Hayaishi (18) presenting evidence of the formation of L-glutamic acid from L-histidine by *Pseudomonas fluorescens*. As far as we are aware, there has been no demonstration previous to that described here of the formation of glutamic acid from histidine in mammalian liver *in vivo* or *in vitro*.

SUMMARY

1. Radioactive glutamic acid was isolated by mild procedures from the liver of animals presented with carboxyl- C^{14} -histidine, in *in vivo* and *in vitro* experiments. The C^{14} was not in the α -COOH group and was very probably in the γ -COOH. This finding supports the mechanism involving urocanic acid, formylisoglutamine, and isoglutamine as intermediates.

2. A radioactive metabolic product of histidine was isolated by ion exchange chromatography whose properties corresponded to those of isoglutamine.

3. The rate of the conversion of histidine to glutamic acid indicates that it is a major metabolic pathway.

BIBLIOGRAPHY

1. Edlbacher, S., and Neber, M., *Z. physiol. Chem.*, **224**, 261 (1934).
2. Featherstone, R. M., and Berg, C. P., *J. Biol. Chem.*, **146**, 131 (1942).

3. Crookshank, H. R., and Clowdus, B. F., *J. Biol. Chem.*, **184**, 307 (1950).
4. Tesar, C., and Rittenberg, D., *J. Biol. Chem.*, **170**, 35 (1947).
5. D'Iorio, A., and Bouthillier, L., *Extrait rev. canad. biol.*, **9**, 388 (1950).
6. Takeuchi, M., *J. Biochem.*, Japan, **34**, 4 (1941).
7. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **196**, 669 (1952).
8. Stein, W., and Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1949).
9. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
10. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
11. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).
12. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).
13. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).
14. Delluva, A. M., and Wilson, D. W., *J. Biol. Chem.*, **166**, 739 (1946).
15. Dent, C. E., *Biochem. J.*, **41**, 240 (1947).
16. Oyamada, Y., *J. Biochem.*, Japan, **36**, 227 (1942).
17. Walker, C. A., and Schmidt, C. L. A., *Arch. Biochem.*, **5**, 445 (1944).
18. Tabor, H., and Hayaishi, O., *J. Biol. Chem.*, **194**, 171 (1952).